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Comparison of Clinical Samples and Methods in Chronic Cutaneous Leishmaniasis

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Abstract. This study aimed at finding out the most effective clinical samples and methods in chronic cutaneous leishmaniasis (CCL). Smear, aspiration fluid, and filter paper samples were taken from 104 skin lesions of suspected cases with CCL, and they were compared using microscopic examination, culture, and molecular methods. We characterized four different forms of CCL and identified the causative agents in CCL forms using high-resolution melting curve real-time polymerase chain reaction assay. We observed that smear was detected to be the most sensitive (63.5%) among clinical samples, and real-time polymerase chain reaction method was the most sensitive (96.8%) among the methods used in diagnosis of CCL. We identified 68.8% *Leishmania tropica* and 31.2% *L. infantum* in papular lesions, 69.2% *L. infantum* and 30.8% *L. tropica* in nodular lesions, 57.9% *L. tropica* and 42.1% *L. major* in ulcerating plaque lesions, and 55.5% *L. tropica* and 44.5% *L. major* in noduloulcerative lesions in CCL patients.

INTRODUCTION

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis, causing a range of skin lesions that may leave lifelong scars.[1] It usually tends to heal spontaneously during a period of 1–2 years depending on the infecting species of *Leishmania*.[1,2] Resolution of the lesion results in a depressed, sometimes disfiguring scar.[2] CL is also classified as acute (< 12 months), chronic (> 12 months), and recidivans according to the duration of the lesion.[3] Long-standing (12 months or longer) cutaneous lesions are defined as chronic CL (CCL).[4] It is clinically classified as papular, nodular, ulcerating plaque, and noduloulcerative according to the appearance of the lesion.[5] Clinical progression and appearances are mostly dependent on the *Leishmania* species.[5,6] CL generally starts as an erythematous papule that develops after an incubation period ranging between 1 and 8 months.[6] This papule gradually enlarges and develops into a nodule over weeks, and this nodule eventually ulcerates in a period of 1–6 months.[6] The volcanic noduloulcerative morphology is the most distinctive feature of CL.[7] It has a painless necrotic base and indurated margin, and its crater is frequently covered by a firmly adherent crust.[7] Some lesions remain at certain stages and never develop into other forms.[6,7]

CCL is easily misdiagnosed by clinical criteria, because the lesions are often atypical, giving rise to terms like carcinoma-like lupoid and sporotrichoid.[2,8] Moreover, because of the low density of *Leishmania* in the chronic stage, conventional laboratory methods alone do not provide accurate results in detecting the protozoan in lesions, the period of which is more than 12 months.[2] Molecular methods, such as polymerase chain reaction (PCR) and real-time PCR methods, are the highly sensitive and specific methods used in the diagnosis of CCL.[9–12] The sensitivity of PCR and real-time PCR is correlated with the copy number of the amplified gene, the primer, and the variety of clinical samples.[13,14]

Keeping in mind the characteristics of CCL, this study was conducted to find out the most effective clinical samples and methods to be used in the diagnosis of CCL. Thus, smear, aspiration fluid, and filter paper samples were taken from the skin lesion of CCL-suspected cases and cross-compared using microscopic examination, culture, and molecular methods. In addition, a relationship between the characterization of clinical features and the causative agents in CCL was investigated using high-resolution melting curve real-time PCR assay.

MATERIALS AND METHODS

Patient selection. Between October of 2009 and April of 2012, 1,104 clinical samples were taken from CL-suspected cases at the Dermatology Department, Faculty of Medicine, Cukurova University. Approval of the study was obtained from the Ethics Committee of the Faculty of Medicine, Cukurova University. The participants suspected of having CL were informed about the study and given a questionnaire. According to the results obtained by the questionnaire, a classification of existing lesions was done for the following categories: acute CL (ACL) less than 1 year in duration, CCL more than 12 months, and leishmaniasis recidivans (LR) characterized by the development of new lesions in the center or periphery of a scar of a healed acute leishmaniasis lesion.

Lesions were then categorized into four main types according to the clinical features. Elevated erythematous lesions smaller than 0.5 cm in diameter were defined as popular. Elevated deeply seated erythematous lesions larger than 0.5 cm were defined as nodular. Erythematous elevated lesions larger than 1 cm in diameter with ulcer were defined as ulcerating plaque, and nodular lesions with a central crater were defined as noduloulcerative lesions.

Aspiration fluid. Skin lesions were cleaned with 70% ethanol before taking the sample aspiration. A 20-gauge needle and syringe containing 0.1 mL sterile saline was then inserted intradermally into the rotate border of the lesion. The syringe was rotated, and the tissue fluids were gently aspirated into the needle as they were withdrawn.

Smear. The infected skin lesions were cleaned with 70% ethanol. Samples were taken using a sterile scalpel to make an incision in the border of the lesion, and a small amount of material was scraped out.

Filter paper. Sterile Whatman 5-mm filter papers (Whatman House, Maidstone, United Kingdom) were gently patted onto the lesion around the edges of the cuts and allowed to air dry thoroughly.

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Microscopic examination. All of the smears were fixed by dipping in absolute methanol and stained with Giemsa 10% stain, and then, they were examined under a light microscope with magnification at 1,000×. Some of aspiration fluid was also smeared onto a glass slide, fixed with methanol, stained with Giemsa, and examined under a microscope. All of the preparations where amastigote was observed were accepted to be positive, and those preparations where amastigote was not observed were negative.

Culture. Approximately 0.05 mL aspiration fluids were inoculated into a 2-mL sterile tube containing 0.1 mL Novy-MacNeal-Nicolle (NNN) medium supplemented with 10% fetal calf serum (FCS; Sigma Aldrich Chemical, France), antibiotics (penicillin and streptomycin at 50 U/mL), and an antifungal agent (fucytosine). The cultures were incubated at 26°C and observed every week for 1 month. Promastigote-observed cultures were accepted to be positive, and cultures where promastigote was not observed were negative.

Reference strains and DNA extraction. The three different *Leishmania* strains used as reference strains were in GenBank: *L. major* (accession number KJ002553), *L. tropica* (accession number KJ002554), and *L. infantum* (accession number KJ002555). Both positive controls with genomic DNA of *Leishmania* reference strains and negative control without DNA template were included in PCR, genus-specific real-time PCR, high-resolution melting curve real-time PCR, and internal transcribed spacer 1 (ITS1) DNA-sequencing methods. No false positivity or contamination occurred in clinical samples and reagents. The DNA was extracted from all of the clinical samples using the DNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

PCR method. The PCR reactions were performed using the primers 13A (5′-GTGGGGGAGGGGCGTTCT-3′) and 13B (5′-ATTTTACACCAACCCCCAGTT-3′) for *Leishmania* species.[15] A final volume of 25 μL of 75 mM KCl, 0.2 mM each deoxyribonucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), 10 pmol each primer, 2.5 U Taq DNA polymerase (D4545; Sigma), and 0.4 pg/μL DNA is mixed in a reaction tube. The PCR program conditions were as follows: initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 94°C for 45 seconds with a final extension at 72°C for 10 minutes. The amplification reactions were analyzed by bromide staining and visualization under ultraviolet (UV) light. The PCR products were considered to be positive with a fragment of the correct size of approximately 120 base pairs (bp).

Genus-specific real-time PCR method. The genus-specific real-time PCR reaction was conducted in a 25-μL volume containing 0.5 pg/μL DNA, 10 pmol each primers JW11 (5′-CCTATTTTACACCAACCCCCAGT-3′) and JW12 (GG GTAGGGGCGTTCTGCGAAA-3′) that amplify a 120-bp fragment of the minicircle kinetoplastid deoxyribonucleotide acid (kDNA) of *Leishmania*,[16] and 1× QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA). The genus-specific real-time PCR program consisted of warming up to 50°C for 2 minutes and initial denaturation at 95°C for 1.5 minutes, which was followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 65°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. All of the reactions were analyzed using the software provided with the instrument. The average cycle threshold (C_T) values were determined, and the standard curves were calculated using the Rotor-Gene 6.1.93 software.

High-resolution melting curve real-time PCR method. A 265- to 288-bp fragment of the *Leishmania* species within the ITS1 region of the *Leishmania* ribosomal RNA operon was amplified by high-resolution melting curve real-time PCR using the primers ITS-219F (5′-AGCTGGATCATTTTCC GATG-3′) and ITS-219R (5′-ATCGCGACACGTTATGT GAG-3′).[17] The total volume was brought to 20 μL with 10 μL QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA), 0.5 μM each primer, 0.5 μM probes, 0.5 pg/μL DNA, and PCR grade water. The amplification program of 95°C for 3 minutes and 45 cycles at 95°C for 10 seconds, 53°C for 10 seconds, and 72°C for 15 seconds was used. A final high-resolution melting curve analysis was performed by initial denaturation at 95°C for 10 seconds followed by 50°C for 10 seconds and continuous heat at 0.1°C per 1 second to 95°C. The transition rate was 20°C per 1 second, except for the extension and final steps, which had temperature transition rates at 1°C per 1 second and 0.1°C per 1 second, respectively. The melting temperatures (T_m) were 53.0°C ± 0.3°C for *L. major*, 61°C ± 0.2°C for *L. tropica*, and 65.0°C ± 0.2°C for *L. infantum*. Threshold and C_T values were automatically determined by Rotor-Gene 6.1.93 software. The C_T and melting temperature (T_m) data were expressed as the mean standard curve ± SD of the three measurements.

Confirmation of high-resolution melting curve real-time PCR. The results of high-resolution melting curve real-time PCR were confirmed by the *Leishmania* ITS1 DNA sequencing method. The PCR was carried out using the LITSR (5′-CTGGATCATTTTCCGATG-3′) and L5.8S (5′-TGA TACCACTTATCGCACTT-3′) primers.[5] All of the PCR reaction mixtures consisted of 1× PCR buffer (75 mM KCl [pH 8.3], 20 mM Tris-HCl, 1.5 mM $MgCl_2$), 1 U Taq polymerase (Fermantas, Burlington, Canada), 0.2 mM $dNTP_s$ (Fermantas, Burlington, Canada), 0.5 pmol each primer, and 5 μL DNA sample. After the initial denaturation (5 minutes at 94°C), 40 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 54°C, and elongation for 1 minute at 72°C were carried out, and the PCR was terminated by final extension at 72°C for 10 minutes. The PCR products were analyzed in 1% agarose gel by electrophoresis at 100 V in 1× Tris-Boric-ethylenediaminetetraacetic acid (EDTA) buffer (0.04 mM Tris-boric, 1 mM EDTA [pH 8]) and visualized by UV light after being stained with ethidium bromide. The ITS1 PCR products of *Leishmania* isolate sequences were found to be approximately 300–350 bp in length.

PCR products were purified using a SentroPure DNA Purification Kit (Sentromer DNA, Istanbul, Turkey), and they were sequenced with the same combination of primers using the BigDye Terminator V 3.1 Cycle Sequencing Kit (Applied Biosystems) according to the protocol of the manufacturer of the 3730 DNA Analyzer (Applied Biosystems, CA). The sequences obtained were processed using the available GenBank and checked by using basic local alignment search tool (BLAST) analysis software (www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Clinical feature of patients. Bearing in mind that CCL should be verified using laboratory methods, because it is often misdiagnosed as other dermatologic problems during

TABLE 1
The results of clinical characterization of 104 CCL-suspected cases

Clinical feature	Rate of clinical feature, % (N = 104)
Papular (< 0.5-cm diameter; N = 28)	26.9
Nodular (> 0.5-cm diameter; N = 23)	22.1
Ulcerating plaque (> 1-cm diameter with ulcer; N = 29)	27.9
Noduloulcerative (> 1-cm diameter with central crater; N = 24)	23.1

clinical examination, we characterized clinical features and analyzed different methods and clinical samples in only 1,104 CCL-suspected cases. Of 1,104 patients, 705 (63.9%) patients and 104 (9.4%) patients were characterized as ACL and CCL, respectively. LR was found in 92 (8.3%) patients, and among skin diseases, like eczema, it was found in 203 (18.4%) patients. Of CCL-suspected cases, 26.9% (28 of 104) had clinical features of papular skin lesions, 22.1% (23 of 104) had clinical features of nodular skin lesions, 27.9% (29 of 104) had clinical features of ulcerating plaque skin lesions, and 23.1% (24 of 104) had clinical features of noduloulcerative skin lesions (Table 1).

Results of microscopic examination, culture, PCR, and real-time PCR methods. The microscopic examination of aspiration fluids revealed 36.5% (38 of 104) positive and 63.5% (66 of 104) negative in CCL-suspected cases. In addition, 40.4% (42 of 104) of smears were positive, and 59.6% (62 of 104) of smears were found to be negative in CCL-suspected cases (Table 2). In all of the CCL-suspected cases, 30.8% (32 of 104) of aspiration fluids were positive, and 69.2% (72 of 104) of cases were negative in culture methods (Table 2).

As for the PCR method, 49.0% (51 of 104) of aspiration fluids were found to be positive, and 51.0% (53 of 104) of aspiration fluids were found to be negative. Of 104 smears, 60.6% (63 of 104) were found to be positive, and 39.4% (41 of 104) were found to be negative; 45.2% (47 of 104) of filter papers were found to be positive, and 54.8% (57 of 104) of filter papers were found to be negative (Table 2).

According to the real-time PCR method, 51.0% (53 of 104) of aspiration fluids were positive, and 49.0% (51 of 104) of aspiration fluids were negative; of the smears, 63.5% (66 of 104) were found to be positive, and 36.5% (38 of 104) were found to be negative. Of filter papers, 46.2% (48 of 104) were found to be positive, and 53.8% (56 of 104) were found to be negative (Table 2). Figure 1 displays the positivity of laboratory methods for the diagnosis of CCL in groups of both suspected and confirmed cases by Venn diagram.

The establishment of the standard curves used serial dilutions of parasites DNA. The final concentrations of parasite DNA per reaction ranged from 2.3 ng to 0.023 fg (equivalent to 40.280–0.004 parasites/reaction). The sensitivity of smear was detected to be higher than that of the aspiration fluid and filter paper.

Comparison of sensitivities and specificities of microscopic examination, culture, PCR, and real-time PCR methods. The results of microscopic examination were compared with culture, PCR, and real-time PCR; the sensitivity levels were found to be 76.2%, 92.9%, and 95.2%, respectively, and the specificities were 100%, 61.2%, and 58.1%, respectively.

When culture was used as baseline, the sensitivities were found to be 100% for all of the methods, and specificities were 86.1%, 56.9%, and 52.8% in microscopic examination, PCR, and real-time PCR methods, respectively.

Sensitivities and specificities of microscopic examination, culture, and real-time PCR were determined using PCR as baseline. The sensitivity and specificity of microscopic examination were 61.9% and 92.7%, respectively. The sensitivities of culture and real-time PCR methods were found to be 50.7% and 96.8%, respectively, and the specificities were found to be 100% and 87.8%, respectively.

When the sensitivity of real-time PCR was compared, microscopic examination, culture, and PCR were found to be 60.6%, 48.5%, and 92.4%, respectively. In addition, the specificities of real-time PCR with microscopic examination, culture, and PCR were cross-compared and found to be 94.7%, 100%, and 94.7%, respectively. All of the sensitivities and specificities are shown in Table 3.

Results of high-resolution melting curve real-time PCR. Of 104 patients, 66 patients had skin lesions with positive smears by genus-specific real-time PCR. In performing the high-resolution melting curve real-time PCR in these patients, 68.8% *L. tropica* and 31.2% *L. infantum* were identified from papular lesions, 69.2% *L. infantum* and 30.8% *L. tropica* were identified from nodular lesions, 57.9% *L. tropica* and 42.1% *L. major* were identified from ulcerating plaque lesions, and 55.5% *L. tropica* and 44.5% *L. major* were identified from noduloulcerative lesions in CCL patients (Table 4). In addition, *L. tropica* was found to be the dominant species among the papular, ulcerating plaque, and noduloulcerative lesions, whereas *L. infantum* was the dominant species among nodular lesions.

Results of ITS1 DNA sequencing. Each of the clinical samples and reference strains of *Leishmania* species (*L. major*, accession number KJ002553; *L. tropica*, accession number KJ002554; *L. infantum*, accession number KJ002555) was identical to the sequence of *Leishmania* species reported in GenBank. Compared with the previous records in GenBank, we determined 100% similarity between sequences of *Leishmania* isolates from CL cases and *Leishmania* strains in GenBank.

DISCUSSION

CL is a common skin disease in Turkey, especially in the Cukurova region, that affects all ages and both sexes.[18,19] The diagnosis of CCL typically involves clinical samples; however, various clinical samples and parasitologic examination

TABLE 2
Comparison between clinical samples and methods in CCL-suspected cases

	Positive, %				Negative, %			
Clinical samples (N = 104)	ME	Culture	PCR	Real-time PCR	ME	Culture	PCR	Real-time PCR
Aspiration fluid	36.5	30.8	49.0	51.0	63.5	69.2	51.0	49.0
Smear	40.4	0	60.6	63.5	59.6	0	39.4	36.5
Filter paper	0	0	45.2	46.2	0	0	54.8	53.8

ME = microscopic examination.

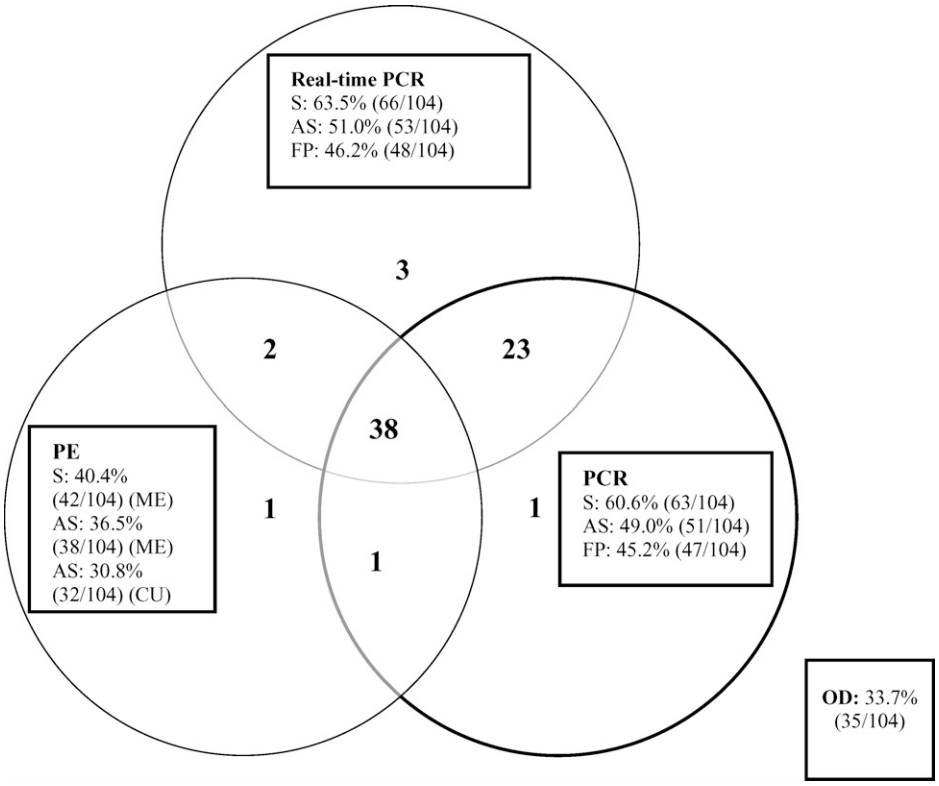

FIGURE 1. Venn diagram with data about PCR, real-time PCR, and parasitologic examination (PE; microscopic examination and culture) positivity regarding CCL-suspected cases ($N = 104$ CCL-suspected cases). The analysis of positivity for PE, PCR, and real-time PCR in patients suspected of CCL shows that one, one, and three patients were positive exclusively in each one of the methods, respectively, and that 38 patients were positive and 35 patients were negative in all three methods. AS = aspiration fluids; CU = culture; FP = filter paper; ME = microscopic examination; OD = other dermatologic diseases; S = smear.

should also be considered, because CCL can be confused with other dermatological problems.[7,20] Furthermore, the causative agent of CCL may affect the treatment. Thus, we took various clinical samples from skin lesions of suspected cases with CCL and cross-compared them using various laboratory methods. In addition, we identified the causative agents in CCL diseases.

The conventional laboratory diagnosis of CCL has limited sensitivity, particularly with the microscopic examination and culture.[21–23] Sensitivity of the microscopic examination has been reported to be ranging from 17% to 83%, and sensitivity of culturing parasites has been reported to be varying from 27% to 85% for diagnosis of CL.[24] In addition, the culture can take days to weeks until parasites are determined depending on the species and number of parasites seeded at the time of the biopsy, and cultures may be contaminated in some cases, reaching 30% of the samples.[24] In this study, we compared the sensitivity and specificity of all the methods, and each method was determined as baseline. We found that the results of our study corresponded with those of the related studies mentioned above, in that the sensitivities of microscopic examination and culture were lower than those of other diagnosis methods. The PCR and real-time PCR methods have recently been reported as having a high sensitivity in the diagnosis of CCL, and the sensitivity of real-time PCR has been suggested to be more than that of the PCR.[25,26] When PCR and real-time PCR methods were compared in diagnosis of CCL, the results of our study confirmed the results of the previous studies.

Skin biopsy samples obtained from the lesions are generally used for molecular diagnosis of CCL.[27] However, the skin biopsy is an invasive and painful procedure.[28] Keeping in mind this fact, we improved the sampling method using smear, aspiration fluid, and filter paper for the diagnosis of CCL in this study, because the collection of these samples was quick, easy, and painless. Aspiration fluid is usually used for culture, and smear is usually used for microscopic examination in routine

TABLE 3
Comparison of methods in CCL

Sensitivity, %				Specificity, %			
ME	Culture	PCR	Real-time PCR	ME	Culture	PCR	Real-time PCR
BL	76.2	92.9	95.2	BL	100	61.2	58.1
100	BL	100	100	86.1	BL	56.9	52.8
61.9	50.7	BL	96.8	92.7	100	BL	87.8
60.6	48.5	92.4	BL	94.7	100	94.7	BL

BL = method was used as baseline; ME = microscopic examination.

TABLE 4
The identification of the causative agents in clinical features of CCL patients

Clinical features ($N = 66$)	*Leishmania* species (%)		
	L. tropica	*L. major*	*L. infantum*
Papular (< 0.5-cm diameter; $N = 16$)	68.8	0	31.2
Nodular (> 0.5-cm diameter; $N = 13$)	30.8	0	69.2
Ulcerating plaque (> 1-cm diameter with ulcer; $N = 19$)	57.9	42.1	0
Noduloulcerative (> 1-cm diameter with central crater; $N = 18$)	55.5	44.5	0

diagnosis of CCL. Filter paper has been used for PCR diagnosis of several diseases, such as visceral leishmaniasis and malaria, but it has never been used for diagnosis of CCL.[29,30] In this study, smear, aspiration fluid, and filter paper were used, and sensitivities of these samples for the diagnosis of CCL were compared using microscopic examination, culture, PCR, and real-time PCR methods. Thus, we assumed that smear, aspiration fluid, and filter paper could be used for the diagnosis of CCL with molecular methods, and smears were the most ideal method. In addition, the sensitivities of real-time PCR methods were more than those of the other laboratory methods in the diagnosis of CCL.

The main cause of CCL is *L. tropica* in the Old World and less commonly, *L. braziliensis* in the New World.[31] *L. major*, *L. tropica*, and *L. aethiopica* have been reported as the causing agents of CCL[32]; the causes are ulcerating plaque lesions, hyperkeratotic lesions, and nodular lesions, respectively.[31,32] The findings of this study correspond with the results of the similar studies. We identified *L. major* as an agent of ulcerating plaque and noduloulcerative lesions. *L. tropica* and *L. infantum* were identified from papular and nodular lesions. In the comparison of species, we also determined that the predominant species in papular, ulcerating plaque, and noduloulcerative lesions was *L. tropica*, whereas *L. infantum* was dominant in nodular lesions. Although many studies reported that the main cause of CL in Turkey is *L. tropica*, we could find very few studies providing satisfactory information about the causative agent of CCL in Turkey before we carried out this study.[33] Thus, using high-resolution melting curve real-time PCR, we described *L. major* as a causative agent of CCL in Turkey. However, more studies are needed to confirm this issue.

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REFERENCES

1. Olliaro P, 2012. Standards for cutaneous *Leishmaniasis* clinical trials published. Available at: http://www.who.int/tdr/news/2013/leishmaniasis. Accessed November 21, 2013.
2. Douba MD, Abbas O, Wali A, Nassany J, Aouf A, Tibbi MS, Kibbi AG, Kurban M, 2012. Chronic cutaneous lesihmaniasis, a great mimicker with various clinical presentations: 12 years experience from Aleppo. *J Eur Acad Dermatol Venereol 26:* 1224–1229.
3. Yemisen M, Ulas Y, Celik H, Aksoy N, 2012. Epidemiological and clinical characteristics of 7172 patients with cutaneous leishmaniasis in Sanliurfa, between 2001 and 2008. *Int J Dermatol 51:* 300–304.
4. Daneshbod Y, Oryan A, Davarmanesh M, Shirian S, Negahban S, Aledavood A, Davarpanah MA, Soleimanpoor H, Daneshbod K, 2011. Clinical, histopathologic, and cytologic diagnosis of mucosal leishmaniasis and literatute review. *Arch Pathol Lab Med 135:* 478–482.
5. Azmi K, Nasereddin A, Eregat S, Schnur L, Schonian G, Abdeen Z, 2011. Methods incorporating a polymerase chain reaction and restriction fragment length polymorphism and their use as a 'gold standard' in diagnosing Old World cutaneous leishmaniasis. *Diagn Microbiol Infect Dis 71:* 151–155.
6. Bari AU, 2012. Clinical spectrum of cutaneous leishmaniasis: an overview from Pakistan. *Dermatol Online J 18:* 4.
7. Lopez FA, Hay RJ, 2004. Parasitic worms and protozoa. Burns T, Breathnack S, Cox N, Giffiths C, eds. *Rook's Text of Dermatology*, Vol. 2, 7th Ed. Oxford, United Kingdom: Blackwell Science Ltd., 35.32–35.47.
8. Weigle KA, Labrada LA, Lozano C, Santrich C, Barker DC, 2002. PCR-based diagnosis of acute and chronic cutaneous leishmaniasis caused by *Leishmania (viannia)*. *J Clin Microbiol 40:* 601–606.
9. Shahbazi F, Shahabi S, Kazemi B, Mohebali M, Abadi AR, Zare Z, 2008. Evaluation of PCR assay in diagnosis and identification of cutaneous leishmaniasis: a comparison with the parasitological methods. *Parasitol Res 103:* 1159–1162.
10. Meymandi SS, Bahmanyar M, Dabiri S, Aflatonian MR, Bahmanyar S, Meymandi MS, 2010. Comparison of cytologic giemsa and real-time polymerase chain reaction technique for the diagnosis of cutaneous leishmaniasis on scraping smears. *Acta Cytol 54:* 539–545.
11. Oliveira DM, Lonardoni MV, Teodoro U, Silveria TG, 2011. Comparison of different primers for PCR-based diagnosis of cutaneous leishmaniasis. *Braz J Infect Dis 15:* 204–210.
12. Rodrigues EH, Felinto de Brito ME, Mendonça MG, Werkhauser RP, Coutinho EM, Souza WV, Militao de Albuquerque F, Jardim ML, Abath FG, 2002. Evaluation of PCR for diagnosis of American cutaneous leishmaniasis in an area of endemicity in northeastern Brazil. *J Clin Microbiol 40:* 3572–3576.
13. Chargui N, Bastien P, Kallel K, Haouas N, Akrout FM, Masmoudi A, Zili J, Chaker E, Othman AD, Azaiez R, Crobu L, Mezhoud H, Babba H, 2005. Usefulness of PCR in the diagnosis of cutaneous leishmaniasis in Tunisia. *Trans R Soc Trop Med Hyg 99:* 762–768.
14. Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I, 2003. Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. *J Clin Microbiol 41:* 3147–3153.
15. Belli A, Rodriguez B, Aviles H, Harris E, 1998. Simplified polymerase chain reaction detection of new world *Leishmania* in clinical specimens of cutaneous leishmaniasis. *Am J Trop Med Hyg 58:* 102–109.
16. Nicolas L, Prina R, Lang T, Milon G, 2002. Real-time PCR for detection and quantitation of *Leishmania* in mouse tissues. *J Clin Microbiol 40:* 1666–1669.
17. Talmi-Frank D, Nasereddin A, Schnur LF, Schönian G, Töz SO, Jaffe CL, Baneth G, 2010. Detection and identification of old world *Leishmania* by high resolution melt analysis. *PLoS Negl Trop Dis 12:* e581.
18. Demirel R, Erdoğan S, 2009. Determination of high risk regions of cutaneous leishmaniasis in Turkey using spatial analysis. *Turkiye Parazitol Derg 33:* 8–14.
19. Uzun S, Uslular C, Yucel A, Acar MA, Ozpoyraz M, Memisoglu HR, 1999. Cutaneous leishmaniasis: evaluation of 3074 cases in the Cukurova region, Turkey. *Br J Dermatol 140:* 347–350.
20. Bourreau E, Ronet C, Darcissac E, Lise MC, Sainte Marie D, Clity E, Tacchini-Cottier F, Couppie P, Launois P, 2009. Intralesional regulatory T-cell suppressive function during human acute and chronic cutaneous leishmaniasis due to *Leishmania guyanensis*. *Infect Immun 77:* 1465–1474.
21. Ghasemian M, Maraghi S, Samarbafzadeh AR, Jelowdar A, Kalantari M, 2011. The PCR-based detection and identification of the parasites causing human cutaneous leishmaniasis in the Iranian city of Ahvaz. *Ann Trop Med Parasitol 105:* 209–215.
22. da Silva RN, Amorim AC, Brandao RM, de Andrade HM, Yokoo M, Ribeiro ML, Bartchewsky W, Socorro-Silva A, de Castro JA, do Monte SJ, 2010. Real-Time PCR in clinical practice: a powerful tool for evaluating *Leishmania chagasi* loads in naturally infected dogs. *Ann Trop Med Parasitol 104:* 137–143.

23. Spanakos G, Patsoula E, Kremastinou T, Saroglou G, Vakalis N, 2002. Development of a PCR-based method for diagnosis of *Leishmania* in blood samples. *Mol Cell Probes 16:* 415–420.
24. Bensoussan E, Nasereddin A, Jonas F, Schnur LF, Jaffe CL, 2006. Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. *J Clin Microbiol 44:* 1435–1439.
25. Carson C, Quinnell RJ, Holden J, Garcez LM, Deborggraeve S, Courtenay O, 2010. Comparison of *Leishmania* OligoC-Tes T PCR with conventional and real-time PCR for diagnosis of canine *Leishmania* infection. *J Clin Microbiol 48:* 3325–3330.
26. Ben Abda I, de Monbrison F, Bousslimi N, Aoun K, Bouratbine A, Picot S, 2011. Advantages and limits of real-time PCR assay and PCR-restriction fragment length polymorphism for the identification of cutaneous *Leishmania* species in Tunisia. *Trans R Soc Trop Med Hyg 105:* 17–22.
27. Jirku M, Zemanova E, Al-Jawabreh A, Schönian G, Lukes J, 2006. Development of a direct species-specific PCR assay for differential diagnosis of *Leishmania tropica*. *Diagn Microbiol Infect Dis 55:* 75–79.
28. Mimori T, Matsumoto T, Calvopina MH, Gomez EA, Saya H, Katakura K, Nonaka S, Shamsuzzaman SM, Hashiguchi Y, 2002. Usefulness of sampling with cotton swab for PCR-diagnosis of cutaneous leishmaniasis in the New World. *Acta Trop 81:* 197–202.
29. Oliveria FS, Pirmez C, Pires MQ, Brazil RP, Pacheco RS, 2005. PCR-based diagnosis for detection of *Leishmania* in skin and blood of rodents from an endemic area of cutaneous and visceral leishmaniasis in Brazil. *Vet Parasitol 129:* 219–227.
30. Boggild AK, Ramos AP, Valencia BM, Veland N, Calderon F, Arevalo J, Low DE, Llanos-Cuentas A, 2011. Diagnostic performance of filter paper lesion impression PCR for secondarily infected ulcers and nonulcerative lesions caused by cutaneous leishmaniasis. *J Clin Microbiol 49:* 1097–1100.
31. Wolff K, Goldsmith LA, Katz SI, Gilchrest BA, Paller AS, Leffell DJ, 2008. *Fitzpatrick's Dermatology in General Medicine*, section 33. New York: McGraw-Hill Companies, 2001–2007.
32. Bailey MS, Lockwood DN, 2007. Cutaneous leishmaniasis. *Clin Dermatol 25:* 203–211.
33. Toz SO, Culha G, Zeyrek FY, Ertabaklar H, Alkan MZ, Vardarlı AT, Gunduz C, Ozbel Y, 2013. A real-time ITS1-PCR based method in the diagnosis and species identification of *Leishmania* parasite from human and dog clinical samples in Turkey. *PLoS Negl Trop Dis 9:* e2205.